COX-2 expression in fibroblast aggregates as a functional indicator for the anti-inflammatory activity of leukemia patients’ bone marrow-derived hematopoietic cells

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Reciprocal communication between hematopoietic cells and their surrounding bone marrow stroma is crucial for normal progression of hematopoiesis [1]. The control network of soluble and insoluble signals regulates both stromal responses and differentiation of hematopoietic precursors, as well as, maintains the stem cell pool. Unfortunately, also malignant cells utilize this intricate signaling system for the promotion and progression of leukemogenesis [2]. In the inflammatory “cancer microenvironment”, the malignant cells and their stroma are nourished by various growth factors and cytokines [3]. Inflammation has been shown to promote the tumor cells’ survival, proliferation, and metastatic activity, as well as, to increase their resistance to chemotherapeutic agents. On the other hand, it is not well known under which circumstances and how the malignant cells modulate or upkeep the inflammatory process for their needs.

In the present study we analysed the ability of the hematopoietic cell fraction, either isolated from leukemia patients’ bone marrow (BM) samples or as cell lines, to modulate stromal inflammation in vitro. To enable functional testing of those cellular processes that govern the signal-regulatory network in the cancer’s aseptic inflammatory microenvironment, we utilized a fibroblast model of cell-to-cell contact and paracrine signaling-induced inflammation, nemosis. This process is effectively induced in dermal fibroblasts [4-6], and is characterized by a massive induction of cyclooxygenase-2 (COX-2) that serves as a sensitive marker for inflammation. Moreover, nemosis-activation of fibroblasts has been shown to
be associated with production of several cytokines associated with inflammation e.g. IL-1, IL-6, IL-8; cell growth factors like HGF, amphiregulin, and FGF7; as well as, inducers of differentiation: IL-11, LIF, and GM-CSF [5,7,8]. Although this mixture of cytokines is similar to that produced by stromal cells isolated from BM [9] and we have shown bone marrow stromal cells (BMSCs) to react in a similar manner upon aggregation [10], fibroblast reactivity is preferred for sensitive functional in vitro testing due to the prompt and prominent induction of COX-2.

The present study was based on our previous observations showing that human leukemic cells can modulate inflammation in fibroblast aggregates as assessed by dose-dependent suppression of COX-2 expression [11]. Although the previously analysed three cell lines were all able to downregulate COX-2 expression, they significantly differed in their potency. In this study, we further extended this analysis of human leukemic cell lines. Moreover, we also tested human hematopoietic cells isolated from BM and peripheral blood of leukemic patients for their ability to modulate this cell-contact and soluble mediator-induced inflammatory activation of fibroblasts.

Our results extend the profile of human leukemic cell lines to modulate stromal inflammation in terms of mesenchymal COX-2 expression. We show here that this co-culture method is also usable for patient samples, and the cells for the assay can be isolated from the same BM aspirate that is also used for flow cytometry immunophenotyping of hematological malignancies.

Materials and methods

Antibodies. Antibodies used for immunoblotting were: goat anti-COX-2 primary antibody (Ab) (sc-1746, Santa Cruz Biotechnology, CA) and anti-goat alkaline phosphatase-conjugated (V115A, Promega, Madison, WI) secondary Ab. Antibodies for flow cytometry (FACS) were: anti-CD45-phycocerythrin (anti-CD45-PE, 555483, BD Biosciences, San Jose, CA), and anti-CD20-allophycocyanin (anti-CD20-APC, 340908, BD Biosciences).

Cell cultures. Human dermal fibroblasts (MUF 7/1)established from human neonatal foreskin were kindly provided by Dr. Miroslav Pirsel (Cancer Research Institute, Bratislava, Slovakia), and were used from passages 5 to 15. Analysed human leukemic cell lines were as follows: THP-1, acute monocytic leukemia [12]; KG-1, acute myelogenous leukemia [13]; U-937, histiocytic lymphoma [14]; HL-60, acute promyelocytic leukemia [15]; ML-2, acute myeloid leukemia [16]; MOLM-9, chronic myelogenous leukemia [17]; Jurkat, acute T cell leukemia [18]; Daudi, Burkitt’s lymphoma [19]; Ramos, Burkitt’s lymphoma [20]; K-562, chronic myelogenous leukemia [21]; NALM-16, acute lymphoblastic leukemia [22]; U-698, lymphosarcoma [23].

The fibroblasts and all leukemic cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 50 IU/mL streptomycin, and 50 µg/mL penicillin (all from Life Technologies, Gibco, Carlsbad, CA).

Patient samples. BM cells were derived from bone marrow aspirates from the posterior iliac crest of BM transplantation donors and from patients suspected of or diagnosed with a hematological malignancy. The source of patient material used for experimentation was clinical specimen obtained from the Department of Paediatric Oncology, University Children’s Hospital and National Cancer Institute, Bratislava. Samples from healthy donors were from the University of Helsinki Central Hospital, Children’s Clinic, Helsinki, Finland and were taken for research purposes. The material used for each assay was the leftover material of the aspirate after diagnostic flow cytometry. BM hematopoietic and stromal cells were isolated by density gradient on Ficoll-Pague™ Plus (GE Healthcare Bio-Sciences AB, Upplands Väsby, SE). Differential adhesion was employed to separate mesenchymal stromal cells from hematopoietic cells. All samples were collected and processed with permission of the Human Ethics Committee of the National Cancer Hospital, Bratislava.

Formation of fibroblast aggregates (spheroids). Spheroid formation was initiated as described previously [4]. Briefly, U-bottom 96-well plates (Costar, Cambridge, MA) were treated with 0.8% LE agarose (BioWhittaker, Rockland, ME) prepared in sterile water to form a thin film of non-adhesive surface. Fibroblasts were detached from culture dishes by trypsin/EDTA, and a single cell suspension (4 x 10⁴ cells/mL) was prepared in complete culture medium. Thereafter 250 µL aliquots were seeded into individual wells, and the dishes were incubated at +37°C in 5% CO₂ atmosphere. Cell numbers were counted using a Bürker chamber.

Co-culture of hematopoietic cells and fibroblast spheroids. For co-culture experiments the leukemic cell lines or the non-adherent fraction of BM was used 24 h after its isolation. The cells were placed in 96-well plates treated with agarose at indicated concentrations. Fibroblasts were added to each well, and the co-cultures were incubated for the next 72 h. Fibroblast spheroids were separated by gravitational differential sedimentation, and analysed for COX-2 expression by immunoblotting. Isolated non-adherent cells were also analysed for COX-2 expression before and after co-cultivation.

Immunoblotting. Cells were washed with PBS, and lysed in reduced sample buffer (62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 5% β-mercaptoethanol and 0.005% bromophenol blue) supplemented with protease inhibitors (Complete Miniprotease Inhibitor Tablets, Roche, Germany), and incubated at +95°C for 5 min. Lysates were centrifuged at 3000 rpm for 5 min to sediment particulate-insoluble material. Each sample separated by SDS-PAGE (gradient of polyacrylamide 5-15%, 3.5% stacking gel) was then transferred to a nitrocellulose membrane (Schleicher&Suell, Dassel, Germany) in transfer buffer at 150 mA during 18 h. Transfer efficiency was verified by Ponceau-S staining. After blocking in 2.5% low-fat dry milk in TBS (20mmol/L Tris-HCl, 150 mmol NaCl, 0.1% Tween-20, pH 7.5) the membrane was incubated
with primary antibodies followed by the secondary alkaline phosphatase-conjugated antibody. Visualization of protein bands was carried out with the Pierce NBT/BCIP western blotting substrates according to manufacturer’s recommendations (Thermo Fisher Scientific, Rockford, IL). IC$_{50}$ values were calculated using the non-linear curve-fitting algorithm in GraphPad Prism version 5 (GraphPad Software, La Jolla, CA) as specified in our previous study [11].

**Flow cytometry.** After standard cultivation or after co-cultivation with spheroids, the hematopoietic non-adherent cell fraction was analysed by flow cytometry. The cells were incubated on ice with anti-CD45-PE, and anti-CD20-APC, and FACS analysis was performed by FACS Canto II flow cytometer (BD Biosciences, San Jose, CA). Data was analysed using Cytlogic program, version 1.2.1.

**Statistic.** Statistical significance was calculated using a two-sided Mann U Whitney test due to small sample size, and p<0.05 was set as significant.

### Results

Using the methodological approach as described in our recent study [11], we evaluated the anti-inflammatory activity of 6 myelo-monocytic and 6 lymphoblastic cell lines. To initiate the co-culture, the leukemic cells were seeded at a density of 1x10$^3$-3x10$^4$ cells/well, and a constant amount of dermal fibroblasts (1x10$^4$ cells/well) was added (Fig. 1). Fibroblast spheroids were separated and harvested when maximal COX-2 induction was reached after 72 h of co-culture. IC$_{50}$ values were calculated by densitometric analysis of COX-2 band intensity on immunoblots. IC$_{50}$ represents the amount of co-cultured leukemic cells that reduces COX-2 expression in fibroblast spheroids to half when compared to COX-2 expression in spheroids alone. The IC$_{50}$ values calculated for the leukemic cell lines are summarized in the Table 1. As reference the IC$_{50}$ values estimated for the three cell lines in the previous study are presented. The IC$_{50}$ values of myelo-monocytic cells were in the range between 746 (THP-1) to 3519 (KG-1), and the mean IC$_{50}$ value for all six myelo-monocytic cell lines was 2084. In general, the lymphoblastic cell lines exhibited lower capacity to inhibit COX-2 expression than the myelo-monocytic cells. IC$_{50}$ values for lymphoblastic cell lines ranged from 3034 (K-562) to 19692 (NALM-16), and the mean IC$_{50}$ value for these cells was 9350. Taken together, the cells of lymphoblastoid origin were thus 4.5 times less potent to inhibit COX-2 expression in comparison to the cells of myelo-monocytic origin.

Because the assay is based on the evaluation of COX-2 expression in fibroblasts co-cultivated with leukemic cells, it was necessary to exclude any contribution to the levels of COX-2 by tested leukemic cells. We therefore assayed all leukemic cell lines for COX-2 expression before and after their co-cultivation with fibroblast spheroids. None of the cell lines was positive for COX-2 expression (data not shown). We detected COX-2 expression only in the HEL erythroleukemia cell line, which was thus excluded from this study.

We then isolated hematopoietic cells from the unused portions of bone marrow aspirates after immunophenotyping. Mesenchymal cells were separated after gradient centrifugation by adherence to plastic, and the non-adherent hematopoietic fraction was used for further analyses. Careful depletion of mesenchymal cells was necessary because of their natural anti-inflammatory properties [25] even when representing less than 1% of the total population. Although we observed changes in the morphology and composition of the samples after 7 days in culture, the most important fact for the established assay was proved by FACS analysis (as...
Moreover, this enabled us to carry out the adherence-depletion method step with the non-adherent population still remaining representative of that in the fresh sample. We then evaluated the effect of sample culturing time on the COX-2 inhibitory activity of isolated non-adherent cells. Aliquots of cells derived from patient #19 were added to co-cultures with spheroids at different time points after their isolation (24 h, 72 h, 144 h). IC_{50} was 3731 for cells that were used for analysis 24 h after isolation, whereas it was 4791 and 5315 for the same cells analysed at time points 72 h and 144 h, respectively (Fig. 3). Prolonged cultivation of non-adherent cells of another patient with acute myeloid leukemia (AML-M7) showed similar decline of inhibitory activity. Again the highest inhibitory effect was observed when cells were analysed 24 h after isolation (IC_{50} ~ 3731). Further cultivation to 96 h, 120 h and 192 h decreased the inhibitory activity (IC_{50} ~ 5910, IC_{50} ~ 8062, IC_{50} ~ 57707) (data not shown). Taken together with the fact that prolonged culturing influences cell morphology and the cell population composition of the sample, these data suggest that cells should be used for experimentation beginning at 24 h after isolation to enable time-dependent monitoring of the inhibitory effect of the fresh samples and to exclude any further influence from culturing. During the prolonged cultivation no decrease of cell viability was observed.

Figure 2. FACS analysis of non-adherent fraction isolated from a bone marrow sample. The analysis was performed in order to compare composition of interface portion obtained from the specimen of AML patient #19 after cultivation for 24 h. The first analysis (0. day) was performed immediately after density gradient separation and the second one (1. day) after 24 h incubation in culture. Marked subpopulations represent: R1 – lymphocytes, R2 – monocytes, R3 – granulocytes, R4 – pathologic cell population. The CD45-PE antibody was used for the analysis.

Figure 3. Effect of prolonged cultivation of non-adherent hematopoietic cells on their ability to modulate COX-2 expression. Immunoblotting analysis showing inhibitory activity of hematopoietic cells isolated from bone marrow of AML patient (# 19) and then cultured for the indicated time points (24 h, 72 h, 144 h) before being added to co-cultures with fibroblast spheroids and analysed. Spheroids prepared from MUF fibroblasts (10^4 cells/well) were harvested for analysis after 72 h of co-cultivation with the same portions of isolated hematopoietic cells as shown in Fig. 1 (Lane 1 to 6 added cells 1 x 10^3 – 3 x 10^4/well). Lane C - represents control, the expression of COX-2 in uninfluenced spheroids.
In our preliminary work we observed that prolonged culturing of the non-adherent hematopoietic fraction for more than 240 h may lead to spontaneous overgrowth of a B-cell population. At that time point this overgrowth was evident by phase contrast microscopy, and was confirmed by FACS analysis, as shown in Fig. 4. Since our observation was not associated with B-cell malignancies, we considered it important to exclude interference to the assay from overgrowth of B-cells during the experiment. We thus selected to routinely perform FACS analysis of all non-adherent hematopoietic cells after the co-culture with spheroids. CD20 was chosen as a characteristic B-cell marker. We observed no B-cell overgrowth during the course of the 96 h co-culture experiments.

Similarly as in the case of leukemic cell lines, it was necessary to test the possible contribution to COX-2 expression by the sample isolated non-adherent cells. Need of such analysis was supported by evidences from recent studies, which showed that some types of hematological malignancies—such as chronic lymphocytic leukemia, chronic myelogenous leukemia, multiple myeloma, or Hodgkin’s and non-Hodgkin’s lymphoma—may exhibit marginal levels of COX-2 expression [26]. Therefore, the sample of non-adherent cells was analysed by immunoblotting after standard culture, as well as, after their co-culture with spheroids. We observed no COX-2 expression in standardly cultured cells or in cells co-cultured after 72 h (data not shown).

Since our results showed that non-adherent cells isolated from bone marrow have the ability to modulate COX-2 expression, we further investigated to what extent the non-adherent cells derived from peripheral blood could possess this capacity. We simultaneously compared non-adherent cells isolated from bone marrow and peripheral blood of the leukemic patient #1. Non-adherent hematopoietic cells isolated from bone marrow (Fig. 5) showed significantly higher inhibition activity of COX-2 expression (IC$_{50}$ ~ 3334) when compared to the value of cells isolated from peripheral blood (IC$_{50}$ ~ 14013). Moreover, similar results were obtained using cells from peripheral blood of healthy donors, where the IC$_{50}$ values ranged from 32372 to 162109 (data not shown).

Next we quantitatively evaluated and compared the ability of individual samples of non-adherent hematopoietic cells isolated from bone marrow specimen of leukemic patients either at diagnosis or during treatment of the disease. Table 2 shows the patient data, and Fig. 6 summarizes the differences in IC$_{50}$ values between controls and patients with malignancy. The non-adherent cells isolated from samples of patients with...
malignancy showed a mean IC_{50} value of 20591±5074 cells whereas the IC_{50} for controls was 9-fold lower at 2284±601 cells (p<0.01). When comparing the samples obtained at diagnosis to those obtained after treatment—for example for relapse or therapy unresponsiveness—their mean IC_{50} values were 3-fold higher (29046±8070 vs. 9600±2850), but remained statistically not significant due to small sample size.

Discussion

Within the bone marrow stem cell niche, maintenance of the stem cell pool and production of differentiated blood cells are controlled by reciprocal signaling between stromal and hematopoietic cells [1]. These complex and finely tuned signals may be disrupted by cancerous and precancerous cells. The malignant cell clone tilts the signaling network to favour its proliferation, survival, and migration. One of the manifestations of such altered signaling network is micro-environmental inflammation that promotes proliferation, disrupts the maturation sequences, causes cellular activation, and induces a migratory phenotype [3]. Due to its biological complexity and multiplex nature, such signaling by malignant cells is difficult to measure by classical analytical methods. In order to be able to evaluate alterations or disruptions in the microenvironmental signal web, a more comprehensive and repeatable functional model system is required. Such an analytical system of the hematopoietic cell fraction should use a live cell sample, it should be sensitive, and it should mimic all the biological complexity of mesenchymal cell activation.

In this report, our aim was to find out the basic feasibility of a co-culture method of hematopoietic cells and nemosis-activated fibroblasts as a functional biological ex vivo platform, and to evaluate the usefulness of COX-2 induction as a measure of anti-inflammatory activity of the sample’s hematopoietic cell fraction. We show here that a routine normal sample of bone marrow, taken for diagnostic purposes and usually for FACS analysis, is sufficient for further functional ex vivo analysis after depletion of the mesenchymal cell fraction by differential adhesion. The mononuclear cell fraction of the bone marrow sample is preincubated for 24 h to separate adherent and non-adherent cells. The hematopoietic (non-adherent) fraction is then used for further analysis. Co-cultures of hematopoietic cells and stromal cells have been used to enable hematopoietic cell maintenance and to mimic the bone marrow microenvironment. Usually, however, the focus has been on the reactivity of the hematopoietic fraction [27-35], and not so much on the opposite direction from hematopoietic cells on stromal cells [36-38]. We reported previously that the interactions between leukemic cell lines and fibroblast spheroids induce leukemia cell growth arrest and differentiation [7]. In our current approach we used preformed multicellular fibroblast spheroids as the indicator component to mimic stromal reactivity. We have previously shown that expression of COX-2 is induced in spheroids of human fibroblasts [4], and that this cell-to-cell contact-mediated activation is associated with an increase in production of a set of inflammatory modulators [5,7]. This process, nemosis, has been linked to inflammation [6], and has been previously used to mimic the stromal reaction in cancer [7,39,40]. In the current report we utilized the nemosis reactivity of fibroblasts and COX-2 induction as a sensitive biological indicator of anti-inflammatory activity of hematopoietic cells isolated from bone marrow of leukemia patients.

The co-culture setting permits a full range of biological interplay, including complex paracrine signaling and cell contacts, to take place. The method evaluates the dose-dependent effects of hematopoietic bone marrow cells on COX-2 expression in fibroblast spheroids. We observed high variability between patients ranging from potent to almost no...
inhibition, suggesting that the method is feasible in detecting sample property-dependent variations. The inflammatory activation in nemosis is induced by a 3-dimensional cell culture setting and utilizes only biological cell-to-cell signaling without any added external influence. We have shown that induction of fibroblast nemosis involves both cell-cell and paracrine interactions. These components are also altered in leukemic cells due to the disease. Homotypic fibroblast-to-fibroblast cell-cell interactions have been shown [4, 41] to be involved in the induction of COX-2 in spheroids. We have also shown that paracrine signaling, by for example prostaglandins, can modulate the COX-2 induction [4]. In the current method, hematopoietic cells are placed in co-culture with the fibroblast spheroids, and are able to interact both physically and by soluble signaling. It is thus possible that heterotypic interactions between hematopoietic/leukemic cells in the sample and fibroblasts contribute to the modulation of COX-2 induction. Leukemic cells differently express for example integrins [42], connexins [43], and chemokine receptors [44] all of which can interfere with the regula-

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tion of homotypic cell-cell contact-induced intracellular signaling in fibroblasts. Moreover, leukemia cells actively secrete several paracrine mediators, such as growth factors, cytokines, microparticles, and exosomes, [45, 46] that can interfere with the inflammatory COX-2 signal transduction in fibroblast spheroids.

Future work should address the uniformity and quality of the sample, because several sample composition-related aspects may influence its properties. Factors that affect the sample include patient age, pharmacotherapy, and disease type. Also technical variation due to location and technique that is used to harvest the aspirate, initial sample volume, and waiting time before aspirate is forwarded to the laboratory for processing evaluation. On the other hand, it must be stressed that bone marrow aspiration is a routine technique in monitoring leukemia patients, and the accompanying data of FACS analysis can be utilized as further control of sample uniformity.

Based on our current results and on the feasibility to use clinical samples for the assay, we propose fibroblast nemosis as a method for laboratory evaluation and monitoring of leukemia cells’ immunomodulatory activity and their ability to modify inflammatory activation. Fibroblast aggregation involves the two functional components: cell-cell contacts and paracrine interactions that may be specifically altered in leukemia. Based on these similarities, we propose that fibroblast nemosis is a prospective sensitive laboratory tool for evaluation and monitoring of leukemia cells’ ability to modulate inflammation. In conclusion, our data shows that performing co-culture of bone marrow hematopoietic cells with human fibroblast spheroids is feasible in the clinical diagnostic setting, and the biologically-induced and pathogen-unrelated inflammatory reactivity can help evaluate the sample’s ability to modulate inflammation. Because the leukemia-stroma crosstalk in the bone marrow modulates the normal signaling network to favour leukemogenesis, this method can provide useful functional biological data to complement the diagnosis or assist for selection of treatment options in specific types of leukemia.

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